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Citation for published version:

Szabo, N, Zhao, T, Cankaya, M, Theil, T, Zhou, XB & Alvarez-Bolado, G 2009, 'Role of Neuroepithelial Sonic hedgehog in Hypothalamic Patterning' Journal of Neuroscience, vol. 29, no. 21, pp. 6989-7002. DOI: 10.1523/JNEUROSCI.1089-09.2009

Digital Object Identifier (DOI):

[10.1523/JNEUROSCI.1089-09.2009](https://doi.org/10.1523/JNEUROSCI.1089-09.2009)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Neuroscience

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Role of Neuroepithelial *Sonic hedgehog* in Hypothalamic Patterning

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The hypothalamus is a region of the diencephalon with particularly complex patterning. *Sonic hedgehog* (*Shh*), encoding a protein with key developmental roles, shows a peculiar and dynamic diencephalic expression pattern. Here, we use transgenic strategies and *in vitro* experiments to test the hypothesis that *Shh* expressed in the diencephalic neuroepithelium (neural *Shh*) coordinates tissue growth and patterning in the hypothalamus. Our results show that neural *Shh* coordinates anteroposterior and dorsoventral patterning in the hypothalamus and in the diencephalon–telencephalon junction. Neural *Shh* also coordinates mediolateral hypothalamic patterning, since it is necessary for the lateral hypothalamus to attain proper size and is required for the specification of hypocretin/orexin cells. Finally, neural *Shh* is necessary to maintain expression of differentiation markers including survival factor *Foxb1*.

Introduction

The hypothalamus is a ventral forebrain region regulating homeostasis and reproduction. Alterations of hypothalamic development can result in endocrine and metabolic disease (Michaud, 2001; Caqueret et al., 2005). Hypothalamic patterning, however, is not well understood. The embryonic hypothalamus forms the rostral–ventral part of the diencephalon. This is a complex region that, together with the caudal–dorsal diencephalon (prethalamus region), forms the diencephalon–telencephalon junction (DTJ). The adult hypothalamus consists of medial and lateral zones. The medial hypothalamus is a series of neuronal aggregates arranged rostrocaudally into four areas called preoptic, anterior, tuberal, and mammillary (MAM), each of them expressing specific markers. The lateral hypothalamus regulates ingestive, aggressive, and reproductive behaviors; without recognizable neuronal nuclei, it is anatomically and functionally very intricate (Simerly, 2004), and information about its specification and patterning is scarce. Specific lateral hypothalamus subpopulations expressing melanin-concentrating hormone (*Pmch*) and hypocretin (*Hcrt*) are essential for the coordination of sleep–wake cycles and feeding behavior (Burdakov et al., 2005).

Ventral patterning of the nervous system is controlled by signaling protein *Shh*, secreted by non-neural tissues like the prechordal plate and notochord (non-neural *Shh*) (Ericson et al., 1997; Gunhaga et al., 2000; Ingham and McMahon, 2001). However, *Shh* is also expressed by the ventral midline of the neural

tube (neural *Shh*). Neural *Shh* is essential for the coordination of tissue growth and pattern in the midbrain–hindbrain junction and cerebellum (Blaess et al., 2006, 2008). In the diencephalic neuroepithelium, *Shh* shows an intriguing and dynamic pattern of expression with domains in the caudal–dorsal diencephalon [zona limitans interthalamica (ZLI)] and the rostral–ventral diencephalon (hypothalamic domain), which seem strategically situated to influence the formation of the DTJ. In the caudal diencephalon, neural *Shh* from the ZLI specifies the prethalamus (PTh) (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Hirata et al., 2006; Scholpp et al., 2006; Guinazu et al., 2007) and promotes growth and differentiation of specific subdivisions of the thalamus (Szabó et al., 2009). The role of neural *Shh* in the rostral diencephalon (hypothalamus) and DTJ, however, is only starting to be analyzed.

We approached the role of neural *Shh* in hypothalamic development through analysis of a conditional mouse mutant as well as experiments *in vitro*. Our results show that neural *Shh* coordinates tissue growth with anteroposterior (AP) and dorsoventral (DV) patterning in the diencephalon and is essential for the formation of the DTJ. In the lateral hypothalamus, neural *Shh* is required for proper size of this zone and for the specification of hypocretin/orexin cells. Finally, neural *Shh* is necessary to maintain expression of differentiation markers including survival factor *Foxb1*.

Materials and Methods

Mutant mouse lines

Animals were treated in ways that minimize suffering and under authorization Az 32.22/Vo from the “Ordnungsamt der Stadt Göttingen,” according to the German Law of Animal Protection.

Foxb1-Cre line. This line expresses Cre recombinase in the mouse diencephalon (Zhao et al., 2007, 2008), and it is a knock-in–knock-out generating *Foxb1* heterozygous animals. These heterozygotes do not show haploinsufficiency and can be considered identical with wild type (WT) (Dou et al., 1997; Labosky et al., 1997; Alvarez-Bolado et al., 2000).

Received March 5, 2009; revised April 21, 2009; accepted April 23, 2009.

This work was supported by The Max Planck Society. Dr. Ulrike Teichmann and her team took expert care of the mutant colonies.

This article is freely available online through the J. Neurosci. Open Choice option.

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DOI:10.1523/JNEUROSCI.1089-09.2009

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Foxb1-Cre/ROSA26R lineage reporter line. In heterozygous *Foxb1-Cre* embryos carrying ROSA26 (Soriano, 1999) or Z/AP (Lobe et al., 1999) reporter alleles, all cells that express or have expressed *Foxb1*, and any cells derived from them, permanently produce β -galactosidase (ROSA26R) or human placental alkaline phosphatase (hPLAP; Z/AP), labeling the *Foxb1* lineage (Zhao et al., 2007).

Conditional allele of Sonic hedgehog. In this conditional allele, exon 2 of *Shh* is flanked by loxP sites (Dassule et al., 2000; Lewis et al., 2001). Exon 2 encodes approximately one-half of the active N-terminal *Shh* signal, essential for *Shh* function (Mann and Beachy, 2004).

***Shh-c* mutant (Foxb1-Cre/*Shh-fl* conditional mutant).** On crossing *Foxb1-Cre* and *Shh-fl* mice, *Shh* conditional mutant mice are produced. We analyze here only mice heterozygous for *Foxb1-Cre* and homozygous for *Shh-floxed* (*Foxb1-Cre*^{+/−} *Shh-fl*^{−/−}), which we term *Shh-c* mutants. No double homozygotes were used for the analysis.

In *Shh-c* mutants, exon 2 of the *Shh* locus has been deleted in the entire *Foxb1* lineage, including the caudal diencephalon, the posterior ventral hypothalamus, and the diencephalic ventral midline (Zhao et al., 2007, 2008). In *Shh-c* mutants, whenever transcription from the *Shh* locus occurs (in cells of the *Foxb1* lineage), the recombined *Shh* locus produces a truncated, nonfunctional mRNA lacking exon 2. An exon 2 probe exclusively detects the functional *Shh* mRNA (full length).

***Shh* full mutants.** To generate full *Shh* mutants (see below) (see Fig. 1*I,J*), we crossed the *Shh-floxed* (Dassule et al., 2000) with a transgenic mouse line carrying Cre under an ubiquitous promoter expressed in embryonic stem cells (Schwenk et al., 1995), in this way producing full mutant homozygous embryos (*CMV-Cre/Shh-floxed*).

Transfection constructs and electroporation

To test the lack of function of *Shh* without exon 2, we prepared constructs expressing exon 2-deleted *Shh*-IRES-EGFP [green fluorescent protein (GFP)] under the control of CAGGS promoter. We obtained a plasmid containing the full-length *Shh* cDNA (from imaGenes), clone IRAVp968F10144D, and we cloned this cDNA into pXL172 (CAGGS promoter–multicloning site–IRES-EGFP). Next, we used PCR to synthesize an exon 2-deleted *Shh* cDNA. In a first PCR step, we used *Shh* cDNA as template to amplify exon 1 and exon 3 in separate reactions. In each reaction, we used “bridge” primers so that the 3′ primer for exon 1 contained 24 nt of the 5′ end of exon 3, and vice versa. We used the product of this amplification as template in a second PCR step, which produced a final continuous exon 1–exon 3 fragment (i.e., exon 2-deleted), which was also cloned into pXL172. The constructs were confirmed by sequencing.

Embryonic day 10.5 (E10.5) mouse brains were harvested, injected with 2 μ g/ μ l DNA, and then electroporated with a CUY21EDIT Nepagene square wave electroporator (Nepa Gene) and the “tweezer electrodes” (22 V; 50 ms Pon, 950 ms Poff; three pulses). The brains were then cut open and cultured for 48 h (see below, Explants), and then GFP expression was analyzed and photographed, and the explants were fixed and treated for whole-mount *in situ* hybridization (ISH).

ISH and reporter detection

Templates were PCR-amplified (primer sequence available on request) from cDNA (from total RNA from newborn mouse brain plus E10.5–E11.5 embryos). Probes were synthesized using the Roche RNA transcription kit (DIG RNA or Fluorescein RNA Labeling Mix). ISH on whole mount or on cryostat sections has been described often. For double whole-mount ISH, probes were labeled with either digoxigenin or fluorescein. Antibodies were as follows: anti-fluorescein-alkaline phosphatase (Fab fragment) (1:5000) and anti-digoxigenin-alkaline phosphatase (Fab fragment) (1:5000) (both from Roche). Color substrates were as follows: BM Purple, Fast Red, or INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Roche).

Staining for alkaline phosphatase and for β -galactosidase activity were performed as described previously (Koenen et al., 1982; Lobe et al., 1999).

5-Bromo-2′-deoxyuridine labeling

Pregnant mice were intraperitoneally injected with 5-bromo-2′-deoxyuridine (BrdU) (RPN201; GE Healthcare) (50 μ g/g of body weight) either at E10.5 or at E12.5, and the embryos were collected 1 h (E10.5) or 3 h (E12.5) later. We used anti-BrdU antibody M0744 (1:100) (Dako) on 20 μ m cryosections after epitope retrieval (2 M HCl for 30 min at 37°C), with nuclear marker 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen) as a counterstain. We examined and photographed the sections under confocal microscopy and counted BrdU-labeled and -unlabeled cells in the neuroepithelium on two histological sections (per level) at one rostral (anterior hypothalamic) and one caudal (MAM) level in three animals per age and genotype (wild type and *Shh-c*). Then we calculated the labeling index (BrdU-labeled cells as percentage of total cells) (Takahashi et al., 1993; Warren et al., 1999; Ishibashi and McMahon, 2002).

Apoptosis detection

We selected cryostat sections of E10.5 and E12.5 brains at two hypothalamic rostrocaudal levels (anterior hypothalamic region and MAM) in three individuals per genotype and pretreated them with 4% paraformaldehyde (20 min) and proteinase K (1.5 μ g/ml; 5 min) at room temperature and then labeled the apoptotic cells with the ApopTag terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling kit (Millipore) according to the instructions of the manufacturer. We counted absolute number of apoptotic cells per histological section of the hypothalamus (both sides) under high magnification with DAPI as counterstain.

Statistical analysis

ANOVA was performed with Prism software (GraphPad Software).

Explants

E9.5 wild-type mouse brains were cut open along the ventral midline, flattened (neuroepithelial side down) on Millicell-CM membranes (Millipore), and cultured under standard conditions in Neurobasal/glutamine (2 mM)/B-27 (Invitrogen). The following experimental reagents were added: Wnt pathway inhibitor 4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl)benzamide (D4476) (Rena et al., 2004; Bryja et al., 2007) (50 μ M in DMSO; Calbiochem), *Shh* pathway inhibitor cyclopamine (15 μ M in DMSO; Sigma-Aldrich), and Wnt pathway activator LiCl (Klein and Melton, 1996) (10 mM; Sigma-Aldrich). Control experiments were performed in parallel with equal DMSO (NaCl to control for LiCl) concentrations. None of the reagents increased cell death (“Live/Dead” Viability/Cytotoxicity kit; Invitrogen) (data not shown). After 48 h, explants were fixed (4% paraformaldehyde) and processed for ISH. The *Foxb1*-expressing area of MAM neuroepithelium was measured on photographs with the help of CellF 2.6 software (Olympus Soft Imaging Solutions).

Results

Foxb1-driven Cre abolishes *Shh* full-length expression in the anterior ventral neural plate and forebrain

We crossed *Foxb1-Cre* heterozygous mice with *Shh-floxed* (Dassule et al., 2000) mice to obtain *Foxb1-Cre*^{+/−}/*Shh-floxed*^{−/−} animals, termed *Shh*-conditional (*Shh-c*) mutants. We did not use double homozygous animals for the analysis, because a *Foxb1*^{−/−} phenotype could alter the *Shh-c* phenotype. *Foxb1*^{+/−} are normal (see Materials and Methods). *Shh-c* mutants die around the time of birth. The patterning processes analyzed here take place much earlier.

The floxed *Shh* mouse line that we use has previously been used to analyze the role of *Shh* in the development of other organs (Dassule et al., 2000; Lewis et al., 2001; Machold et al., 2003; Komada et al., 2008). On Cre-mediated recombination, the floxed *Shh* loses exon 2 (Dassule et al., 2000; Lewis et al., 2001), which encodes the N terminal, required for *Shh* function (Fan et al., 1995; Hynes et al., 1995; Lai et al., 1995; López-Martínez et al., 1995; Martí et al., 1995; Roelink et al., 1995). However, we wanted

to ascertain that this was also the case in the forebrain neuroepithelium. E10.5 explants transfected with a construct expressing full-length *Shh* expressed ectopic *Ptch1* (diagnostic of *Shh* pathway activation) (for review, see Lewis et al., 2001), whereas transfection of *Shh* constructs in which exon 2 had been deleted did not have any effect (Fig. 1A–E).

To analyze our mutants, we needed to know whether and when the mutant neural plate was able to express *Shh*. At E8.0, *Foxb1* was expressed in the ventral midline of the wild-type anterior neural plate (Fig. 1F) (Zhao et al., 2007), whereas *Shh* was expressed in the prechordal mesoderm but not yet in the neural plate of wild type or mutant (Fig. 1G,H). *Foxb1* was expressed in the neural plate of a *Shh*^{−/−} embryo (full *Shh* knockout mutant), indicating that *Shh* is not required for initiation of *Foxb1* expression (Fig. 1I,J).

At E8.5, both *Foxb1* and *Shh* were expressed in the wild-type ventral neural plate (Fig. 1K,L). In the mutant, the *Shh* locus was active as revealed by ISH with a “long probe” detecting any *Shh* transcripts (i.e., wild-type *Shh* mRNA as well as truncated *Shh* mRNA, the product of a Cre-recombined *Shh* locus) (Fig. 1M), but it produced nonfunctional *Shh* mRNA in the neural plate, as shown by an exon 2 probe, which detects only the product of the wild-type *Shh* locus (non-Cre-recombined) (see Materials and Methods) (Fig. 1N). At the 12–14 somite stage, *Foxb1* and *Shh* were expressed in wild-type ventral diencephalon with a rostral limit at the optic sulcus (Fig. 1O,P, arrowhead) (Shimamura et al., 1995; Zhao et al., 2007). In the mutant, the *Shh* locus was active in appropriate domains (Fig. 1Q), but full-length, functional *Shh* transcripts were limited to the ventral caudal diencephalon (Fig. 1R, arrow), not rostral diencephalon (hypothalamus) (Fig. 1R, arrowhead).

These results indicated complete elimination of functional *Shh* mRNA in the *Shh-c* hypothalamus from E8.5 on.

Activation of *Shh* expression in the basal plate depends on neural *Shh*

Since diencephalic *Foxb1* expression is mostly transient (Zhao et al., 2007, 2008), we used a lineage reporter mouse line to identify the regions in which a phenotype can be expected. In these embryos, cells that have expressed *Foxb1*, as well as cells derived from them, express β -galactosidase permanently, allowing for the identification of the regions in which *Foxb1*-driven Cre recombination has taken place (see Materials and Methods).

At 21–24 somites, *Foxb1*-lineage cells were found in the ventral part of rostral and caudal diencephalon, in the diencephalic ventral midline up to eye levels (Fig. 1S), and in the branchial arches (Fig. 1S, arrowheads). *Shh* at this age had disappeared from the ventral side (Fig. 1, compare P, T) and was expressed in four distinct domains: (1) suboptical, (2) a longitudinal band representing the basal plate (bp) of the rostral diencephalon (Puelles et al., 2004), (3) a small domain in the MAM, and (4) the incipient ZLI in the caudal diencephalon (Fig. 1T). The ventral portion of the tuberal area was devoid of *Shh* expression at this age (Fig. 1T, arrowhead). In *Shh-c* mutants, *Shh* transcriptional activation was abolished in the bp and ZLI domains, but maintained in the MAM and suboptical domains (Fig. 1U). Functional *Shh* was expressed only in the suboptical domain, with reduced intensity (Fig. 1V). In the ZLI domain, maintenance of activity in the *Shh* locus requires *Shh* (feedback loop) (Kiecker and Lumsden, 2004; Zeltser, 2005), which explains the lack of a ZLI in this mutant, and possibly also the lack of a bp domain. In contrast, the suboptical domain and the MAM domain can activate *Shh* expression independently of *Shh* of neural origin.

The major brain regions can be recognized by expression of specific markers at E12.5 (Shimamura et al., 1995). At this age, the thalamus, the MAM, and scattered cells in prethalamus and hypothalamic floor plate were of *Foxb1* lineage (Fig. 1W), indicating that those regions would not be able to express functional *Shh* in the mutant. In the wild-type embryo at this age, a new *Shh* domain was present [medial ganglionic eminence (MGE)] and the domains detected earlier were fully developed (Fig. 1X). The MAM showed an *Emx2*-expressing subdomain corresponding to its most prominent neuronal nucleus, the mammillary body (MBO) (Fig. 1X, inset; supplemental Fig. 1A, available at www.jneurosci.org as supplemental material) and a *Shh*-expressing subdomain. The bp domain of *Shh* expression was still strong in the tuberal area (Fig. 1X). In the *Shh-c* brain, the bp domain was absent (as well as the ZLI) (Fig. 1Y), and most of the remnant *Shh* transcriptional activity produced inactive *Shh* transcripts, except for the MGE (Fig. 1Z).

Early abolition of the *Shh* pathway in the *Shh-c* forebrain

Non-neural sources of *Shh* can still activate the *Shh* pathway in the mutant neuroepithelium. Therefore, we characterized the state of the pathway in *Shh-c* embryos by detecting expression of *Shh* receptor *Ptch1* and transcription factor gene *Gli1*, diagnostic markers of *Shh* pathway activity (for review, see Lewis et al., 2001). At E8.5, the pathway was active in the mutant ventral midline (Fig. 2A–D), probably because of a response to non-neural *Shh* from the prechordal plate. At 14 somites, *Gli1* and *Ptch1* expression in the mutant disappeared from the ventral diencephalon (Fig. 2E–H) but was retained in the ventral telencephalon further demonstrating the specific ablation of *Shh* in the diencephalon (Fig. 2H). The loss was maintained at 24 somites (Fig. 2I–L). These results show a shift in the dependence of the wild-type neural plate from non-neural to neural sources of *Shh* and indicate that, in the *Shh-c* neural plate, the *Shh* pathway is rendered inactive very early at diencephalic levels.

Regional hypothalamic patterning in the *Shh-c* embryo

Shh is essential to specify the hypothalamus (Chiang et al., 1996). Regional specification of the hypothalamus is defined by *Nkx2-1* (*Titf1*) expression (Kimura et al., 1996; Puelles et al., 2004). We found *Nkx2-1* expression in an appropriate (although reduced in size) region of the *Shh-c* forebrain at E9.5 (Fig. 3A,B) and E11.5 (Fig. 3C,D), indicating the presence of a ventral forebrain region fated to become hypothalamus.

The major brain regions can be recognized by specific marker expression at E12.5 (Shimamura et al., 1995). To explore the regionalization of the hypothalamus in the absence of neural *Shh*, we detected expression of *Dlx2* (Pierani et al., 2001; Petryniak et al., 2007) and *Dbx1* (Lu et al., 1992; Shoji et al., 1996), transcription factor genes expressed in the presumptive hypothalamus and with important developmental roles. (*Nkx2-1* at E12.5 can be seen in Fig. 4A,B.) Expression of *Dlx2* (Fig. 3E,F) and *Dbx1* (Fig. 3G,H) was almost completely abolished in the mutant hypothalamus.

The MAM in the *Shh-c* embryo

Dlx2 expression in the mutant (Fig. 3F) suggests abolition of the PTh. This could be expected, since neural *Shh* (from the ZLI) is essential for PTh development, as has been thoroughly documented (see references in Introduction). However, the continuity of dorsal and ventral diencephalon in the absence of a PTh has not been investigated. Rostral to the ZLI, the diencephalon consists of PTh and thalamic eminence [eminencia thalamica

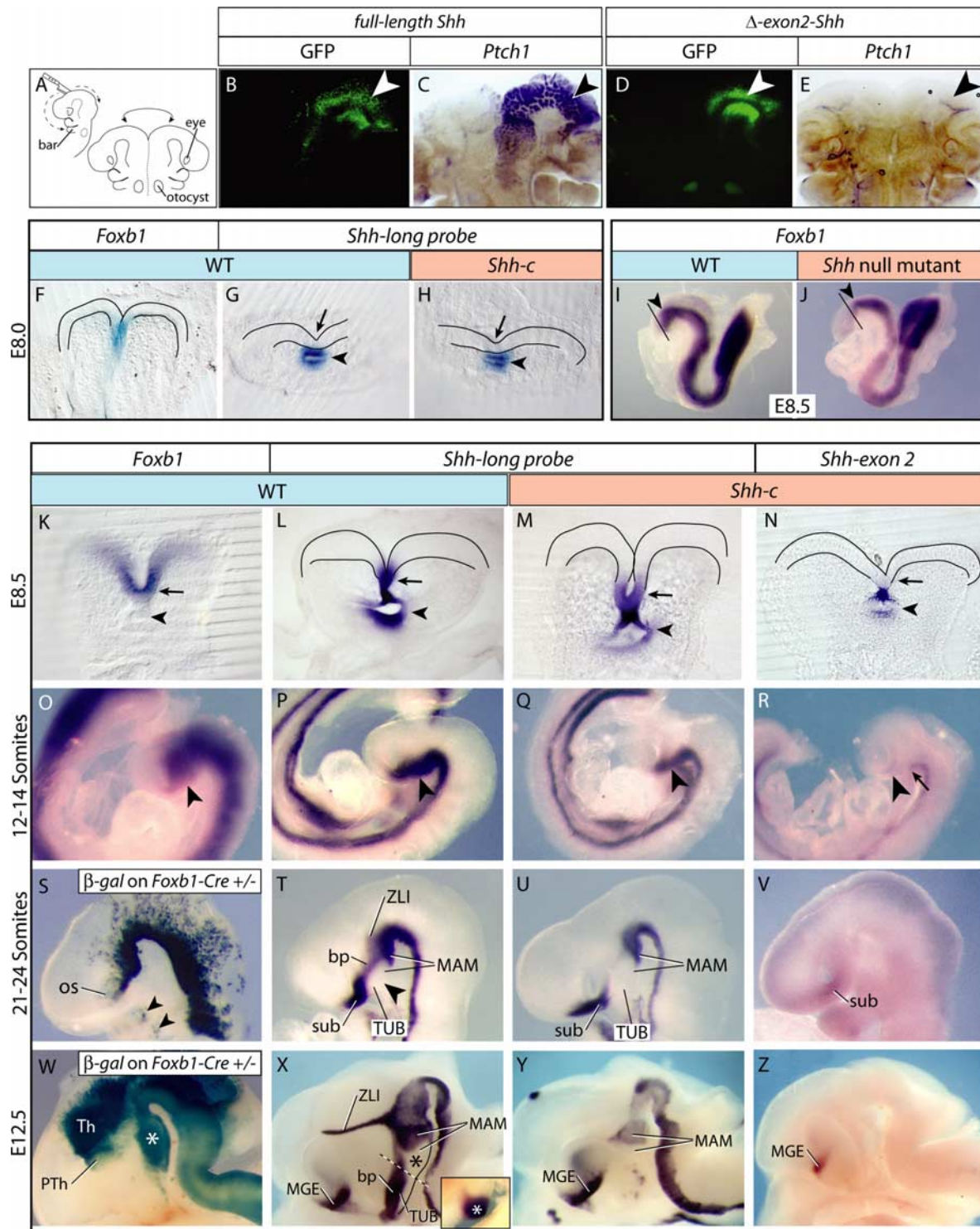


Figure 1. Foxb1-driven Cre abolishes *Shh* expression in the anterior ventral neural plate and forebrain. **A–E**, Explant cultures according to **A** of E10.5 wild-type brains (**B–E**) electroporated with full-length *Shh* (**B, C**) or exon 2-deleted *Shh* (**D, E**). Transfected areas (**B, D**, white arrowheads) express GFP. Full-length *Shh* induced ectopic *Ptch1* expression (**C**) but exon 2-deleted *Shh* did not (**E**). **F–H**, ISH for *Foxb1* (**F**) and *Shh*-long probe (**G, H**) on E8.0 anterior neural plate in wild type (**F, G**) and *Shh-c* (**H**). The ventral neural plate shows *Foxb1* (**F**), but not yet *Shh* in wild type (**G**) or mutant (**H**). **I, J**, *Foxb1* is expressed in the early neural plate in wild type (**I**) and *Shh*^{−/−} (full mutant) (**J**). The arrowhead indicates the diencephalon. The black line indicates the DTJ. **K–N**, At E8.5, *Foxb1* and *Shh* are coexpressed in wild-type ventral anterior neural plate (**K, L**, arrow). *Shh* is expressed in foregut also (**K, L**, arrowhead). In the mutant, *Shh* is expressed in the ventral rostral neural plate (**M**, arrow) and foregut (**M**, arrowhead) but lacks exon 2 in the neural plate (**N**, arrow), not foregut (**N**, arrowhead). **O–R**, At 12–14 somites, *Foxb1* (**O**) and *Shh* (**P**) overlap in ventral forebrain (**O, P**, arrowhead). In the *Shh-c*, *Shh* was expressed in a comparable domain (**Q**, arrowhead), but lacked exon 2 (**R**, arrowhead). The tegmentum expressed some full-length *Shh* (**R**, arrow). **S–V**, β-Galactosidase shows *Foxb1* lineage in *Foxb1-Cre/ROSA26R* mice at 21–24 somites (**S**) overlapping with *Shh* expression (**T**) (arrowheads in **S** show *Foxb1*-lineage cells in the branchial arches). *Shh* expression (**T**) was absent from ventral forebrain (arrowhead) and showed novel domains: suboptical, basal plate (bp), ZLI, and MAM. In the mutant, *Shh* transcriptional activation was missing in the bp and ZLI domains (**U**). Functional *Shh* transcripts were produced only in the sub, in reduced amounts (**V**). **W–Z**, At E12.5 the *Foxb1* lineage covers thalamus and most of the prethalamus (Th and PTh in **W**), as well as MAM (**W**, asterisk). *Shh* expression (**X**) showed a telencephalic domain (MGE). Dotted line, TUB–MAM boundary. In the MAM, the MBO (**X**, asterisk) does not express *Shh* but *Emx2* (**X**, inset) (see also supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). In the mutant (**Y**), only the MAM and MGE domains remained. Only the MGE produced functional *Shh* transcripts (**Z**). For the abbreviations used in the figures, see Table 1.

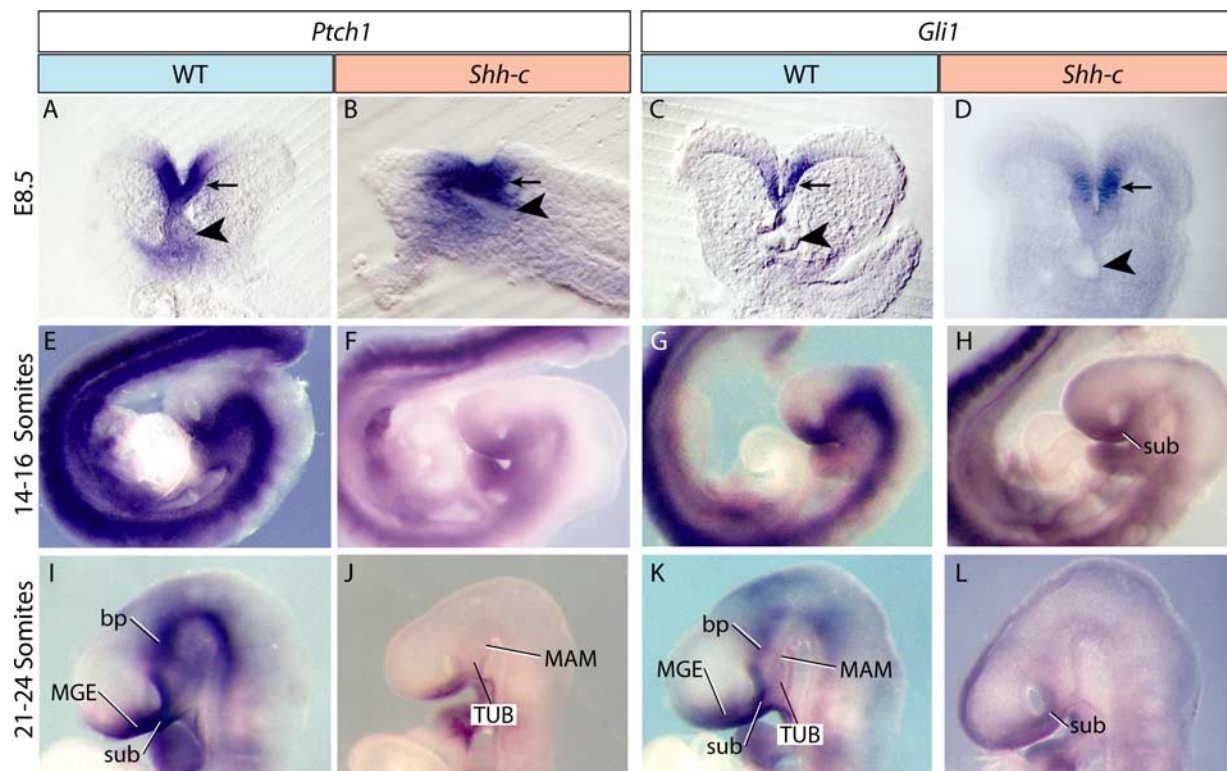


Figure 2. The *Shh* pathway is gradually abolished in the early *Shh-c* forebrain. *In situ* hybridization for *Ptch1* and *Gli1* on wild-type and *Shh-c* mutant embryos. **A–D**, At E8.5, *Ptch1* and *Gli1* were expressed in the ventral rostral neural plate (arrow) in wild type (**A**, **C**) and mutant (**B**, **D**). The arrowheads indicate the foregut. **E–H**, At 14–16 somites, both *Ptch1* and *Gli1* were expressed in the entire ventral neural tube (**E**, **G**) but had disappeared from the mutant rostral neural tube (**F**, **H**) except in the sub. **I–L**, At 21–24 somites, the domain of expression of *Ptch1* and *Gli1* in wild type (**I**, **K**) showed domains similar to *Shh*: bp and sub, and an incipient telencephalic domain, MGE (compare with Fig. 17), whereas in the mutant both markers were still absent from the forebrain (**J**, **L**) except for low-intensity expression of *Gli1* in the sub (**L**).

(EMT)]. *Lhx5* labels specifically the EMT and the MBO, but not the PTh (Bachy et al., 2001) (Fig. 3I). In the mutant, an abnormal *Lhx5* domain encompassed a stripe of dorsal and ventral diencephalon (Fig. 3J), as if formed by a fusion between EMT and MAM. Colocalization of this marker with *Pitx2*, a specific marker of the MAM (Skidmore et al., 2007), showed that the MAM is not dorsally expanded in the mutant, but the absence of PTh tissue leads to abnormal contiguity of EMT and MAM (Fig. 3K, L).

Wnt8b expression labels the MBO in all vertebrates (Cui et al., 1995; Kelly et al., 1995; Lako et al., 1998; Garda et al., 2002; Houart et al., 2002), as well as the rostral border of the dorsal diencephalon (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). In the *Shh-c* mutant, both domains coalesce (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material) like the *Lhx5* domains (Fig. 3J). Codetection of *Wnt8b* with *Pitx2* (supplemental Fig. 1D, E, available at www.jneurosci.org as supplemental material) confirmed that both domains become contiguous in the mutant, as opposed to a dorsal expansion of the MAM. The fact that *Lhx5* is expressed in a mutant without PTh represents a genetic dissection of the rostral–dorsal diencephalon. We analyzed this by colocalizing EMT marker *Tbr1* (Puelles et al., 2000) and PTh marker *Arx* (Kitamura et al., 1997), which show contiguous domains in the rostral–dorsal diencephalon (supplemental Fig. 1F, available at www.jneurosci.org as supplemental material). As expected, *Arx* was missing in the mutant, but *Tbr1* also (supplemental Fig. 1G, available at www.jneurosci.org as supplemental material). [The wild-type expression patterns of *Foxg1*, *Foxd1*, *Tbr1*, and *Pitx2* as single

markers can be found in supplemental Fig. 1H–K (available at www.jneurosci.org as supplemental material).]

The apparent fusion of rostral–dorsal diencephalon with the MAM results in an alteration of the DTJ. The expression domains of transcription factor genes *Foxd1* and *Foxg1* define the DTJ (Hatini et al., 1994; Herrera et al., 2004) (Fig. 3M). Codetection of these markers in the mutant was less informative, since *Foxd1* failed to be expressed (Fig. 3N). However, careful examination of the DTJ in our material showed that, in the mutant, it goes all the way to the ventral side, as shown in the diagrams in Figure 3, O and P.

The DTJ in the *Shh-c*

To examine the DTJ more closely, we sectioned E12.5 wild-type and mutant embryos along the horizontal plane of section (indicated in Fig. 4A, B, K, L) and labeled them for hypothalamic marker *Nkx2-1* as well as MBO marker *Emx2* (Fig. 4A, B, K, L). *Nkx2-1* labeling showed that the mutant tuberal area was much shorter along the anteroposterior axis (Fig. 4E–H) and had lost *Nkx2-1* expression in a large caudal area (Fig. 4C–J).

The most dorsal expression of *Nkx2-1* in the hypothalamus (Fig. 4A, arrow) marked a depression or sulcus in the neural tube (Fig. 4C, arrow), which could be followed to ventral levels in the wild type (Fig. 4C, E, G, arrows) and the mutant (Fig. 4D, F, H, J, arrows).

Emx2 expression labeled the MBO very specifically in the wild type (Fig. 4K, S; supplemental Fig. 1A, available at www.jneurosci.org as supplemental material), and in the mutant it was present in an abnormal caudal structure (Fig. 4L, arrowhead). *Emx2*-labeled sections of the mutant showed that the sulcus mentioned above starts at the point (Fig. 4N, arrow) at which the

Table 1. Abbreviations

Abbreviation	Definition
3V	Third ventricle
ac	Anterior commissure
AHA	Anterior hypothalamic area
bar	Branchial arch
bp	Basal plate
CTX	Cortex
DG	Dentate gyrus
DTJ	Diencephalon–telencephalon junction
EMT	Thalamic eminence (eminencia thalamica)
hPLAP	Human placental alkaline phosphatase
Hy	Hypothalamus
LH	Lateral hypothalamus
MAM	Mammillary area
MBO	Mammillary body
MGE	Medial ganglionic eminence
Os	Optic sulcus
oto	Otocyst
PCP	Prechordal plate
PRO	Preoptic area
PT	Prethalamus
PTh	Prethalamus
PVN	Paraventricular nucleus
SC	Superior colliculus
SCH	Suprachiasmatic nucleus
sub	Suboptical domain
TG	Tegmentum
Th	Thalamus
TL	Telencephalon
TUB	Tuberal area
VL	Lateral ventricle
VMH	Ventromedial nucleus
ZLI	Zona limitans interthalamica

cortex (telencephalon) meets the diencephalon, in coincidence with the beginning of an *Emx2*-expressing hypothalamic region which seems an extension of the cortex (Fig. 4*N*, arrowhead). The sulcus could be followed in the ventral direction in the wild type (Fig. 4*M*, *O*, *Q*) and mutant (Fig. 4*N*, *P*, *R*, *T*).

However, in the *Shh-c* hypothalamus, *Emx2* expression labeled a region flattened rostrocaudally and with a convoluted lumen (Fig. 4*N*, *P*, *R*, *T*, arrow and arrowhead) more reminiscent of cortex than hypothalamus.

An *Emx1*-expressing layered structure in the ventral diencephalon of the *Shh-c* embryo

Emx1 is a very specific cortical marker not expressed in any other structure (Gulisano et al., 1996) and labels the entire cortex of the E12.5 mouse (Fig. 5*A*, *B*). In the *Shh-c* mutant, intriguingly, the *Emx1*-expressing cortex extended toward the midline (Fig. 5*C*, *D*, red arrowheads). In coronal sections (Fig. 5*E–H*) of wild type and mutant, we could ascertain expression of *Emx1* in one layered structure occupying the ventral side of the diencephalon (Fig. 5*G*, *H*, red arrowheads). Detection of *Emx1* at E18.5 (Fig. 5*J*, *K*) confirmed this finding (Fig. 5*K*, red arrowheads). *Tbr1* is a marker of the pallium (cortex and hippocampus) and thalamic eminence (Bulfone et al., 1995) and it was expressed in a layered structure positioned in the mutant hypothalamus at E18.5 (Fig. 5*L*, *M*). *Foxg1* (Xuan et al., 1995) is a telencephalic marker (Fig. 5*N*) also expressed in this structure (Fig. 5*O*, arrow).

Cell proliferation and cell death in the *Shh-c* hypothalamic neuroepithelium at E10.5 and E12.5

Shh promotes proliferation and cell viability in the diencephalic neuroepithelium at E9.0 (Ishibashi and McMahon, 2002). We

wanted to know whether neural *Shh* controls those cellular processes in the hypothalamus after that age. To address this question, we analyzed *Shh-c* mutant embryos for proliferation and cell death. We analyzed proliferation by BrdU labeling at E10.5 (1 h survival) and E12.5 (3 h survival). At E10.5, we did not find any difference in labeling index between wild type and mutant (data not shown). At E12.5, however, we found in rostral sections a moderate (~20%) but significant ($p < 0.001$) decrease in proliferation in the mutant (labeling index in rostral hypothalamus: 49.5 ± 0.70 in wild type vs 40 ± 4.20 in the mutant; in caudal hypothalamus: 50 ± 0.5 in wild type vs 51.5 ± 0.70 in the mutant) (data not shown). We did not find changes in cell death in the hypothalamus at E10.5 or E12.5 (data not shown).

The ventral diencephalon of the *Shh-c* is transversally divided into two parts

Next, we wanted to assess diencephalic differentiation at a later stage, when neurogenesis is over and the mantle layer is differentiated. Since *Shh-c* mutants do not survive beyond the end of gestation, we analyzed E18.5 brains. Inspection of Nissl-stained sections showed size reduction of the basal ganglia, hypothalamus and thalamic region, and an enlarged third ventricle (Fig. 6*A*, *B*). As expected from previous results (Fig. 5*K*), a layered structure (Fig. 6*B*, asterisk) was positioned medially and ventrally. To identify the major brain regions in the mutant, we used marker genes *Gbx2*, *Nkx2-1*, *Lhx1*, and *Calb1*. *Gbx2*, a thalamic transcription factor (Miyashita-Lin et al., 1999), identified a reduced mutant thalamus (Fig. 6*C*, *D*). Expression of *Nkx2-1*, an essential marker of the early hypothalamus as a region, is restricted during differentiation to specific hypothalamic nuclei including part of the preoptic area and the mammillary body (Price et al., 1992) (Fig. 6*E*). Expression of *Nkx2-1* was maintained in the mutant preoptic area (Fig. 6*F*). Immediately caudal to the preoptic area is the anterior hypothalamic area, a prominent nucleus of which is the suprachiasmatic (SCH), specifically expressing transcription factor *Lhx1*. *Lhx1* expression was preserved in the mutant (Fig. 6*G*, *H*). The next rostrocaudal subdivision, the tuberal region, contains the ventromedial nucleus (VMH), specifically expressing *Calb1* (Fig. 6*I*). In the mutant, the VMH was smaller but expressed the marker (Fig. 6*J*). The layered structure was positioned immediately caudal to the VMH in the mutant (Fig. 6*J*) but rostral to the MBO (Fig. 6*F*, *H*). This suggested that this structure separated the preoptic, anterior, and tuberal areas from the MAM.

These results are summarized in Figure 6, *K* and *L*. Additional information can be found in supplemental Figure 2 (available at www.jneurosci.org as supplemental material).

Neural *Shh* is required for the development of the lateral hypothalamus

To explore the mediolateral extension of the mutant hypothalamus we detected expression of arginine vasopressin (*Avp*), which encodes a hormone specifically expressed in the paraventricular (PVN) and supraoptic (SO) nuclei (Fig. 7*A*). In the mutant, the PVN was reduced, *Avp* neurons were scattered in the mediolateral plane and the SO was absent (Fig. 7*B*). However, on sagittal sections labeled with specific marker genes *Sim1* (Michaud et al., 1998) and *Otp* (Acampora et al., 1999) (supplemental Fig. 2*E–H*, available at www.jneurosci.org as supplemental material), the PVN was not expanded rostrocaudally. Since the hypothalamus is strongly patterned in the transverse plane (i.e., medial hypothalamus vs lateral hypothalamus), these data suggested a specific alteration of mediolateral patterning.

In horizontal sections, the lateral hypothalamus can be recog-

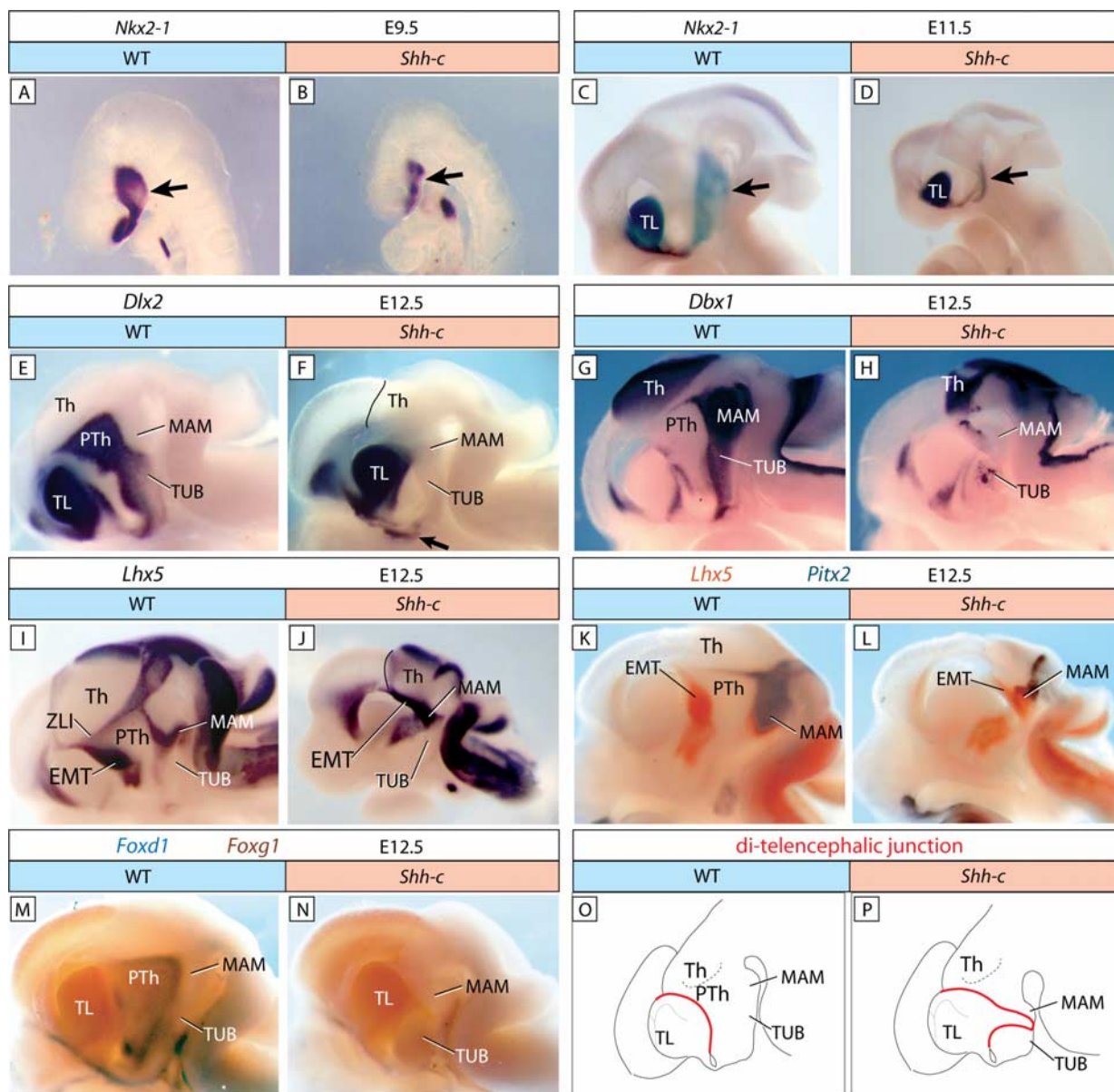


Figure 3. Loss of hypothalamic markers in the *Shh-c* embryo. **A–N**, Whole-mount ISH detection on hemisected embryo brains. Ages, probes, and genotypes are as indicated. **A–D**, At E9.5 (**A, B**) as well as E11.5 (**C, D**), *Nkx2-1* was expressed in the ventral forebrain in wild type (**A, C**, arrows) and mutant (**B, D**, arrows). **E, F**, The TUB (and PTh) domains of *Dlx2* (**E**) were almost completely abolished in the mutant (**F**) (the arrow indicates a remnant). The upper border of Th is outlined for clarity. **G, H**, The hypothalamic domain of *Dbx1* expression (**G**) (MAM plus TUB) was absent in the mutant (**H**). **I, J**, The MAM and EMT domains of *Lhx5* (**I**) are reduced and seem fused in the mutant (**J**). **K, L**, Colocalization of *Lhx5* and *Pitx2* expression clarifies the extent and position of the MAM with respect to the EMT in the mutant. **M, N**, *Foxg1* and *Foxd1* show the DTJ in wild type (**M**). In the mutant, *Foxd1* is not expressed (**N**). **O, P**, Diagrams showing the DTJ (red line) in wild-type (**O**) and *Shh-c* (**P**) E12.5 hemisected brains as seen in the previous panels.

nized by the large number of longitudinal axons that course through it, as opposed to the more compact medial hypothalamus (Fig. 7C). By this criterion, a lateral hypothalamus seems to be mostly absent in the mutant (Fig. 7D). By crossing our *Shh-c* conditional mutants with the mouse reporter line Z/AP (Lobe et al., 1999), we labeled the neurons of *Foxb1* lineage (see Materials and Methods), which are very abundant in the lateral hypothalamus (Fig. 7E) but were dramatically reduced in the mutant (Fig. 7F). Specific expression of marker genes *pro-melanin-concentrating hormone* (*Pmch*) (Fig. 7G) and *hypocretin-orexin* (*Hcrt*) (Fig. 7I) characterizes two independent neuronal subpopulations in the lateral hypothalamus. The number of *Pmch* neurons was extremely reduced in the mutant (Fig. 7H), whereas *Hcrt* neurons were completely absent (Fig. 7J). These results

(summarized in Fig. 7K,L) show that deficiency in neural *Shh* results in alterations of growth and terminal differentiation of the lateral hypothalamus as well as in the lack of specific, functionally important neuronal subpopulations.

Neural *Shh* is required to maintain expression of the mammillary survival factor *Foxb1*

Dependence of *Foxb1* expression on neural *Shh* (Fig. 8A,B) was surprising, since *Foxb1* is expressed in the *Shh*-deficient neural plate (Fig. 1I,J), suggesting a feedback loop between both factors.

We analyzed this question on cultured explants of whole embryonic forebrain. For this purpose, we dissected the forebrains of wild-type E9.5 mouse embryos, cut them open following the ventral midline, and flattened them on Millipore membranes

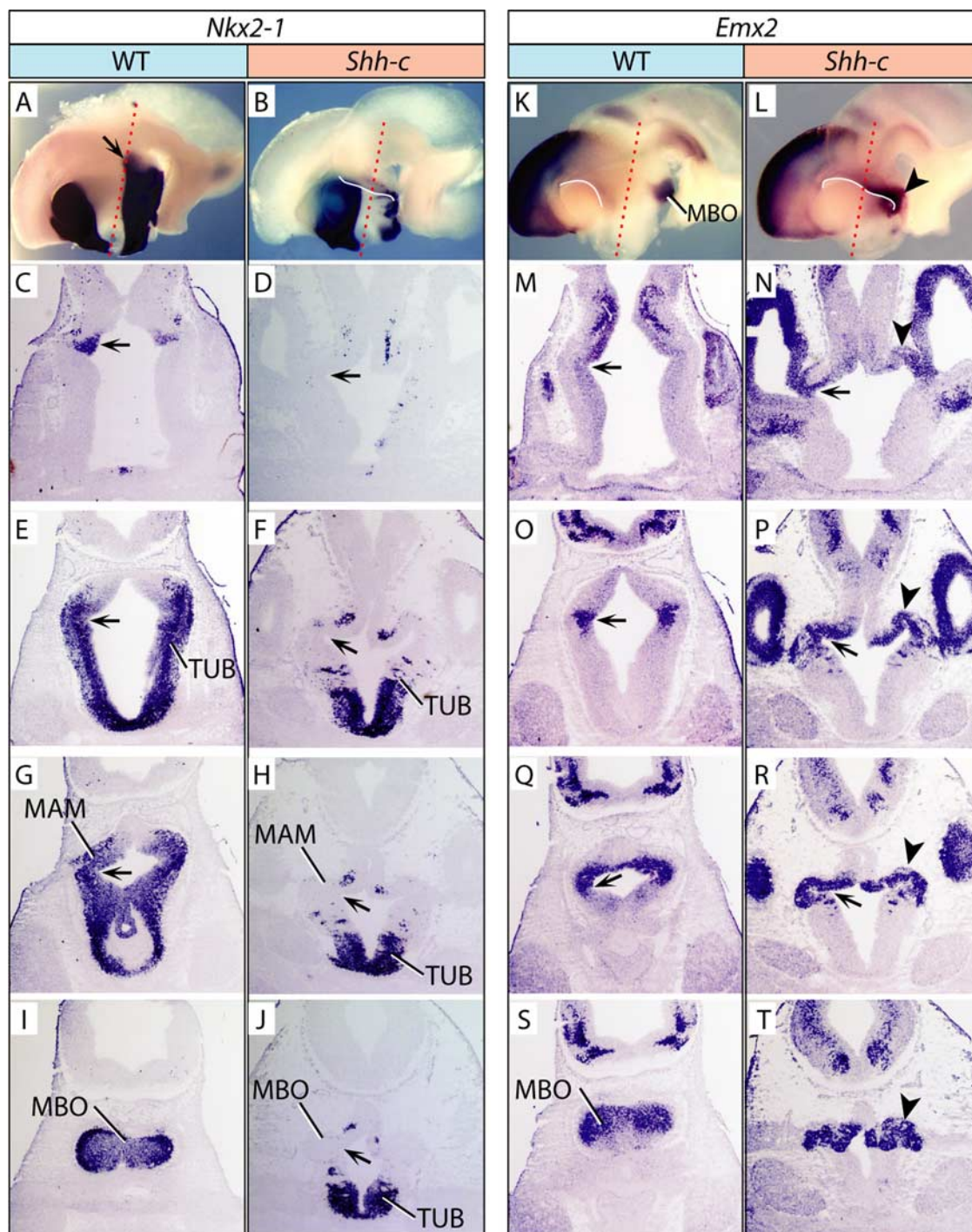


Figure 4. The *Shh-c* hypothalamus in horizontal sections at E12.5. **A, B, K, L**, Whole-mount detection of gene expression on hemisected E12.5 brains; probes and genotypes are as indicated. The red dotted lines indicate approximate plane of section of the panels below. The white lines in **B, K**, and **L** emphasize the DTJ. **C–J, M–T**, ISH detection of *Nkx2-1* (**C–J**), and *Emx2* (**M–T**) on horizontal sections showing four dorsal-to-ventral levels of the hypothalamus of WT and *Shh-c* E12.5 brains as indicated. The most dorsal hypothalamic domain of *Nkx2-1* (**A, C**, arrows) in the wild type marks a sulcus that can be followed up to ventral levels in wild type and mutant (**D–H, J, M–R**, arrows). **M, N**, *Emx2* expression shows that the sulcus corresponds in the mutant to the point at which diencephalon and telencephalon meet (**N**, arrow), in close proximity to a domain of *Emx2* expression (**N**, arrowhead). This *Emx2* domain can be followed in the mutant from the DTJ (**N**) to ventral levels of the hypothalamus (**P, R, T**). **S**, Expression of *Emx2* in the wild-type MBO. **T**, A convoluted structure expresses *Emx2* in the mutant.

with the neuroepithelial side down (Fig. 8C–E). The explants were cultured for 48 h in control media or after adding one of several reagents to modify the activity of different signaling pathways. *Shh* pathway inhibitor cyclopamine abolished *Foxb1* expression in E9.5 explants (in 8 of 10 explants; 8 of 10) (Fig. 8F, G), but not in E12.5 explants (9 of 11) (Fig. 8H, I). Therefore, between E9.5 and E12.5, neural *Shh* maintains *Foxb1* expression,

perhaps against inhibiting influences by other factors. A powerful signaling agent, *Wnt8b*, specifically expressed in the MBO (Fig. 8J–L) is able to reduce the *Foxb1* MAM expression domain in zebrafish (Kim et al., 2002). We therefore tested whether canonical Wnt signaling controls *Foxb1* expression. Wnt pathway activator LiCl (Stambolic et al., 1996) decreased the *Foxb1* MAM expression domain (9 of 10) (Fig. 8M, N). D4476 is a casein

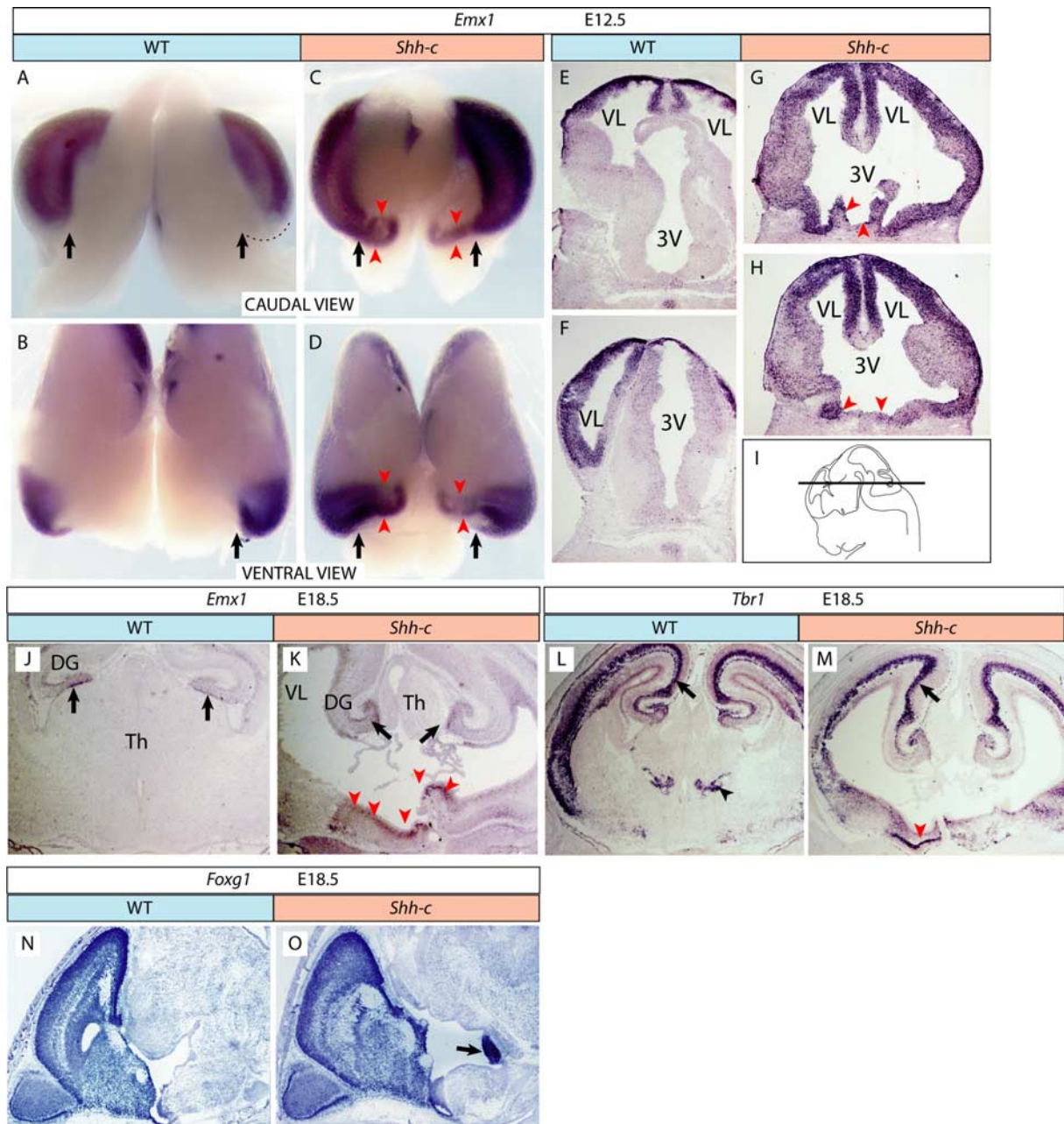


Figure 5. Layered structure in the ventral diencephalon in the *Shh-c* brain. **A–D**, E12.5 wild-type (**A**, **B**) and *Shh-c* (**C**, **D**) brains hybridized for *Emx1* in caudal (**A**, **C**) and ventral (**B**, **D**) view (the diencephalic ventral side was cut open longitudinally). The black arrows indicate the medial limit of the cortex in wild type (**A**, **B**) and comparable point in the mutant (**C**, **D**). The red arrowheads indicate ectopic expression of *Emx1* in the mutant (**C**, **D**). **E–I**, Sections of E12.5 wild-type (**E**, **F**) and *Shh-c* (**G**, **H**) brains hybridized for *Emx1* show ventral ectopic expression in the mutant (**G**, **H**, red arrowheads). The plane of section is shown in **I**. **J**, **K**, *Emx1* expression on sections of E18.5 wild-type (**J**) and *Shh-c* (**K**) brains. The black arrows indicate normal expression in hippocampus. The red arrowheads (**K**) indicate neuroepithelial ectopic *Emx1* expression in the mutant. **L**, **M**, *Tbr1* expression on sections of E18.5 wild-type (**L**) and *Shh-c* (**M**) brains. The black arrows indicate normal expression in hippocampus. The black arrowhead in **L** indicates EMT expression of *Tbr1* in wild type. The red arrowhead in **M** indicates one well defined layer of ectopic *Tbr1* expression in the mutant. **N**, **O**, *Foxg1* expression labels the telencephalon (**N**, **O**) as well as the ectopic structure in the mutant (**O**, arrow).

kinase-1 inhibitor able to block the Wnt pathway (Rena et al., 2004; Bryja et al., 2007). In explants cultured with D4476 the *Foxb1* MAM expression domain increased in size (9 of 11) (Fig. 8O–Q). These results suggested that the Wnt pathway, probably activated by *Wnt8b*, restricts the *Foxb1* MAM expression domain in the neuroepithelium. In agreement, as *Wnt8b* expression increases in the MAM neuroepithelium during development, *Foxb1* is downregulated in the neuroepithelium and upregulated in the newly born MBO neurons; by E12.5 the separation of the expression domains of both markers in the MAM was complete

(Fig. 8R,S). These results suggest that neural *Shh* maintains *Foxb1* MBO expression, whereas *Wnt8b* inhibits it in the neuroepithelium, thereby restricting *Foxb1* to neurons. Our results, together with some data from the literature (see Discussion), are summarized in Figure 8T.

Discussion

By analyzing a neuroepithelium-specific *Shh* mutant phenotype, we elucidate the differential roles of *Shh* from the notochord and the neuroepithelium on hypothalamic development. We show

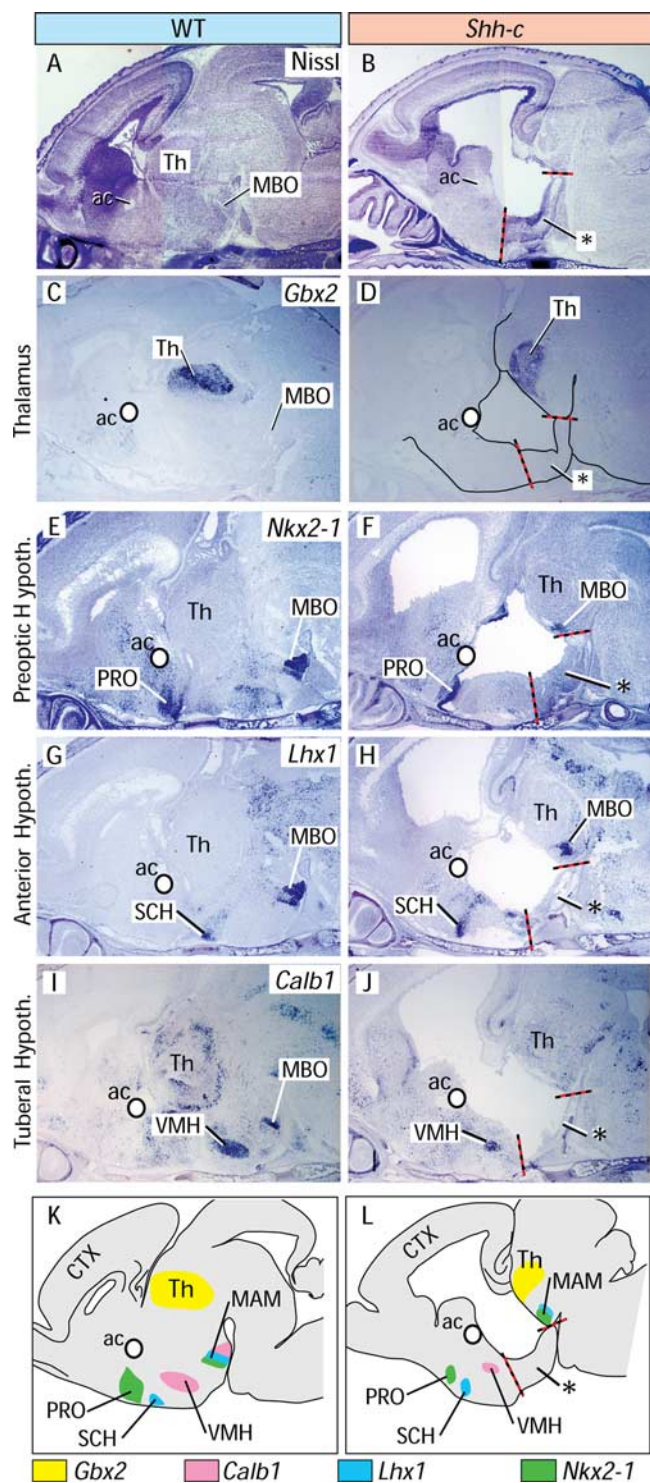


Figure 6. Neural *Shh* is required for differentiation in the medial hypothalamus. **A, B**, Nissl-stained sagittal sections of E18.5 wild-type (**A**) and mutant (**B**) brains. The red–black line indicates the boundaries of the abnormal layered structure (asterisk) in **B**. **C–J**, ISH on sections of wild-type (**C, E, G, I**) and *Shh-c* (**D, F, H, J**) E18.5 brains. A white circle marks the anterior commissure for reference. **C, D**, *Gbx2* labels the thalamus and was preserved in the mutant. **E, F**, Expression of *Nkx2-1* is preserved in the mutant PRO and MBO. **G, H**, *Lhx1* is preserved in the mutant SCH and MBO. **I, J**, *Calb1* is preserved in the mutant VMH but lost in the MBO. **K, L**, Summary diagram of results.

that neural *Shh* is essential for the coordination of tissue growth and acquisition of AP and DV patterning in the hypothalamus and the DTJ. We demonstrate a role for neural *Shh* in growth and pattern coordination along the mediolateral axis of the hypothal-

amus as well as in the specification of lateral hypothalamic neuronal subpopulations. We further show that neural *Shh* differentially promotes cell proliferation in the rostral and MAM regions. In addition, neural *Shh* influences the maintenance of *Foxb1* expression in postmitotic MBO neurons by interplay with the Wnt pathway.

Finally, since our mutant expresses *Nkx2-1* in the ventral diencephalon and has two eyes, our results imply that neural *Shh* is dispensable for hypothalamic regional specification and eye field separation, two processes depending on *Shh* signaling (Chiang et al., 1996; Mathieu et al., 2002).

Neural *Shh* in hypothalamic precursor expansion and neurogenesis

Shh is needed early for proliferation in the diencephalon (Ishibashi and McMahon, 2002) and in particular in the caudal regions (Manning et al., 2006), which agrees with the reduced size of the hypothalamus in our mutant already at E9.5 (Fig. 3*B*). Our data indicate that this requirement is temporally restricted, and by E10.5 it has disappeared. However, we show that neural *Shh* has some promoting effect on proliferation in the rostral hypothalamus at E12.5, during the most active phase of hypothalamic neurogenesis (Altman and Bayer, 1986; Clancy et al., 2001). The effect of neural *Shh* on the neuroepithelium at this age could be direct, since weak expression of *Gli1* and *Ptch1* can be detected at ventral levels of the hypothalamic neuroepithelium through E12.5 and persists at least until E14.5 (data not shown). The effect of neural *Shh* could also be indirect, as in the early dorsal diencephalic proliferation (Ishibashi and McMahon, 2002).

Shh is necessary for cell viability in the diencephalic neuroepithelium at E9.0 (Ishibashi and McMahon, 2002) and in the mid-brain–hindbrain junction until E10.5 (Blaess et al., 2006). We show, however, that in the hypothalamus this effect is temporally restricted, and it has disappeared by E10.5.

Neural *Shh* and AP patterning in the hypothalamus

In *Shh-c* brains, reduced growth of the medial hypothalamus causes the hypothalamic mantle to become abnormally separated into a rostral subdivision (the three rostral hypothalamic regions) and a caudal one (the MAM). The gap that is created in this way unexpectedly confirms neural plate fate-mapping data in zebrafish showing that the presumptive MAM is indeed caudal to, but not continuous with the rest of the hypothalamus (Staudt and Houart, 2007). This region has been considered a somewhat atypical part of the hypothalamus from the point of view of specification mechanisms (Mathieu et al., 2002), patterning (Puelles and Rubenstein, 2003), connectivity (Thompson and Swanson, 2003), and function (Stackman and Taube, 1998; Vann and Aggleton, 2004; Radyushkin et al., 2005).

In addition, the mutant MAM is extremely reduced and becomes contiguous with the thalamic eminence, altering the DV pattern of the diencephalon (see below).

Neural *Shh* in the PTh and EMT

It has been well documented that deficiency in neural *Shh* from the ZLI leads to abolition of the PTh (see citations in Introduction). Therefore, our mutant lacks a PTh, as reflected by the loss of *Dlx2* (Fig. 3*E, F*) and *Arx* expression (supplemental Fig. 1*F, G*, available at www.jneurosci.org as supplemental material). However, *Lhx5*, a specific marker of the EMT, is preserved in our mutant, suggesting that lack of neural *Shh* in this region performs a genetic dissection between the two regions [*Tbr1*, another specific marker of the thalamic eminence, is however lost in the

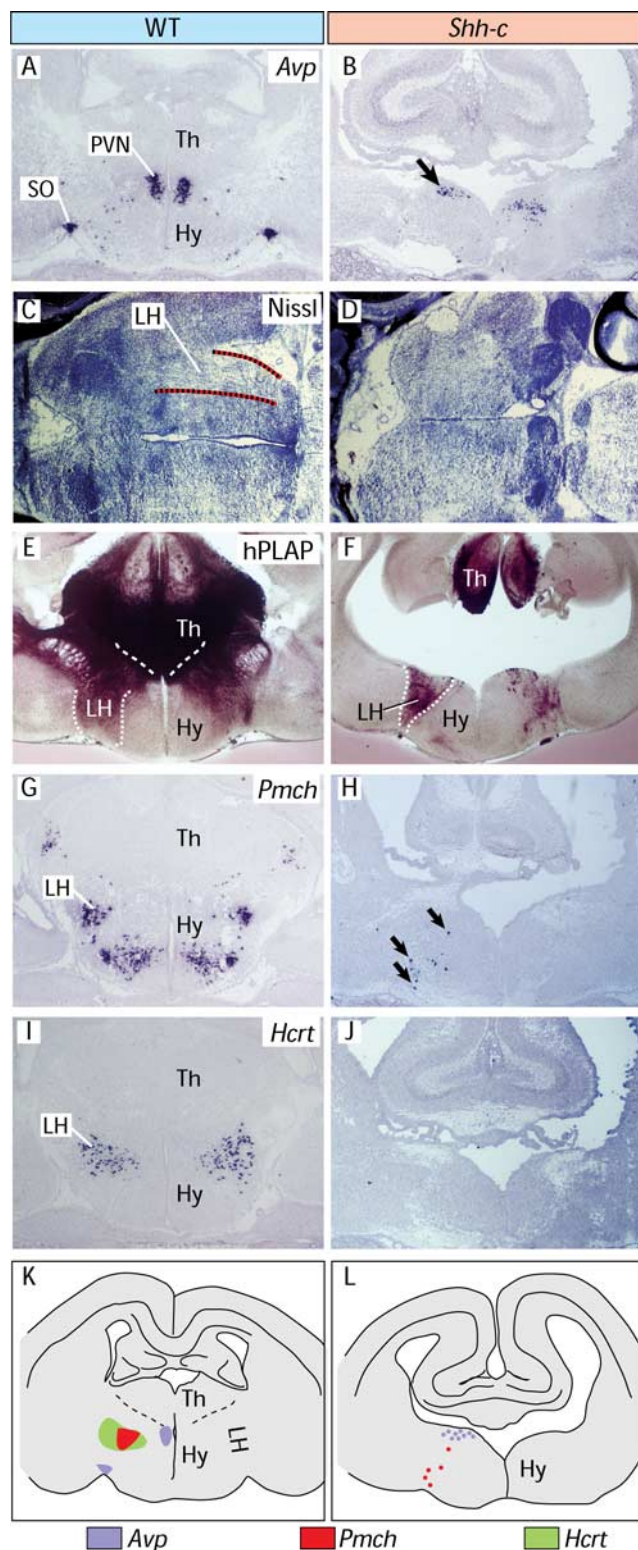


Figure 7. Neural *Shh* is required for lateral hypothalamus development. **A, B**, ISH on transverse sections of E18.5 shows that the *Avp* expression domain is broader in the mutant and the SO is absent. **C, D**, Horizontal sections through E18.5 wild-type (**C**) and mutant (**D**) brains. The lateral hypothalamus (delimited with dotted lines in **C**) shows typically abundant longitudinal axons. A similar structure cannot be found in **D**. **E, F**, hPLAP detection of the *Foxb1* lineage in *Foxb1-Cre/ZAP* reporter mice (see Materials and Methods) shows the normal extent of the lateral hypothalamus (**E**), very reduced in the *Shh-c* mutant (**F**). **G, H**, *Pmch* neurons are very scarce in the mutant. **I, J**, *Hcrt* neurons cannot be detected at all in the mutant. **K, L**, Summary diagrams of results. Expression is shown on the left side of each diagram, and abbreviations are on the right side.

mutant (supplemental Fig. 1 F, G, available at www.jneurosci.org as supplemental material)]. This reduces the size of the dorsal diencephalon and enlarges the communication between the lateral ventricles and the third ventricle.

Neural *Shh* coordinates AP and DV patterning in the diencephalon and the formation of the DTJ

Surprisingly, the patterning defects in dorsal and ventral diencephalon compound to alter the DTJ, which in the mutant extends ventrally to reach the midline (Fig. 3O,P). This is reminiscent of the major role played by neural *Shh* in the coordination of the AP and DV patterning in the midbrain–hindbrain junction (Blaess et al., 2006). In wild-type embryos, the growth of the thalamus and hypothalamus forces the expanding medial pallium to acquire a characteristic curved shape (compare, for instance, the hippocampus of wild type and mutant in Fig. 5L,M). In the *Shh-c* embryo, the very reduced hypothalamus (and thalamus) does not oppose resistance to the pallium, which stretches unimpeded toward the midline, particularly on the ventral side (Fig. 5A–D). At later stages, this fact has as an unexpected consequence the presence of part of the pallium (presumably the hippocampus) mispositioned at the caudal end of the ventral hypothalamus in the mutant.

Neural *Shh* in mediolateral hypothalamic patterning

Mediolateral patterning of the hypothalamus involves the specification of its distinctive medial and lateral zones. Between E9.5 and E13.0 approximately, *Shh* is expressed as a longitudinal band in the basal plate of the ventral hypothalamus (Shimamura et al., 1995). This expression domain coincides in time (E10.3–E12.7) (Altman and Bayer, 1986) and space (Altman and Bayer, 1986; Marchand et al., 1986), with the primordium of the lateral hypothalamus. We show that abolition of *Shh* expression in the hypothalamic neuroepithelium results in a very reduced lateral hypothalamus in which some of the most functionally important and characteristic neuronal subpopulations are either very reduced (*Pmch* neurons) or completely missing (*Hcrt/orexin* neurons). This indicates that neural *Shh* has a very important and specific role in the development of the lateral hypothalamus, possibly mediated by regulation of *Dlx2*, *Dbx1*, and *Foxd1* (Fig. 3).

An interplay between *Shh* and *Wnt8b* regulates expression of *Foxb1*, a crucial regulator of caudal hypothalamus development

Deficiency in neural *Shh* translates also in incomplete differentiation of hypothalamic nuclei. We used the MBO as model to analyze this defect, since in the mutant this nucleus expresses several specific differentiation markers (*Nkx2-1*, *Lhx1*), but fails to express others like *Sim1*, *Otp*, and *Foxb1*. The case of *Foxb1* is particularly intriguing, since it is expressed by the neural plate (E8.5) in the *Shh* full mutant (Fig. 1I,J) but fails to be expressed at E18.5 in the *Shh-c* MBO (Fig. 8A,B). Our explant experiments show that, at E12.5, the dependence on *Shh* has disappeared. Since *Foxb1* is still missing in the mutant MBO at E18.5, we conclude that neural *Shh* is necessary for maintenance of *Foxb1* expression.

Incidentally, since *Foxb1* is essential for the survival of the MBO neurons beyond the day of birth (Alvarez-Bolado et al., 2000), this result reveals an unexpected requirement for neural *Shh* in differentiation and survival of the MAM and is in keeping with data showing a role of *Shh* in neuronal survival in other systems (Miao et al., 1997; Oppenheim et al., 1999).

We also show that Wnt signaling suppresses *Foxb1* expression

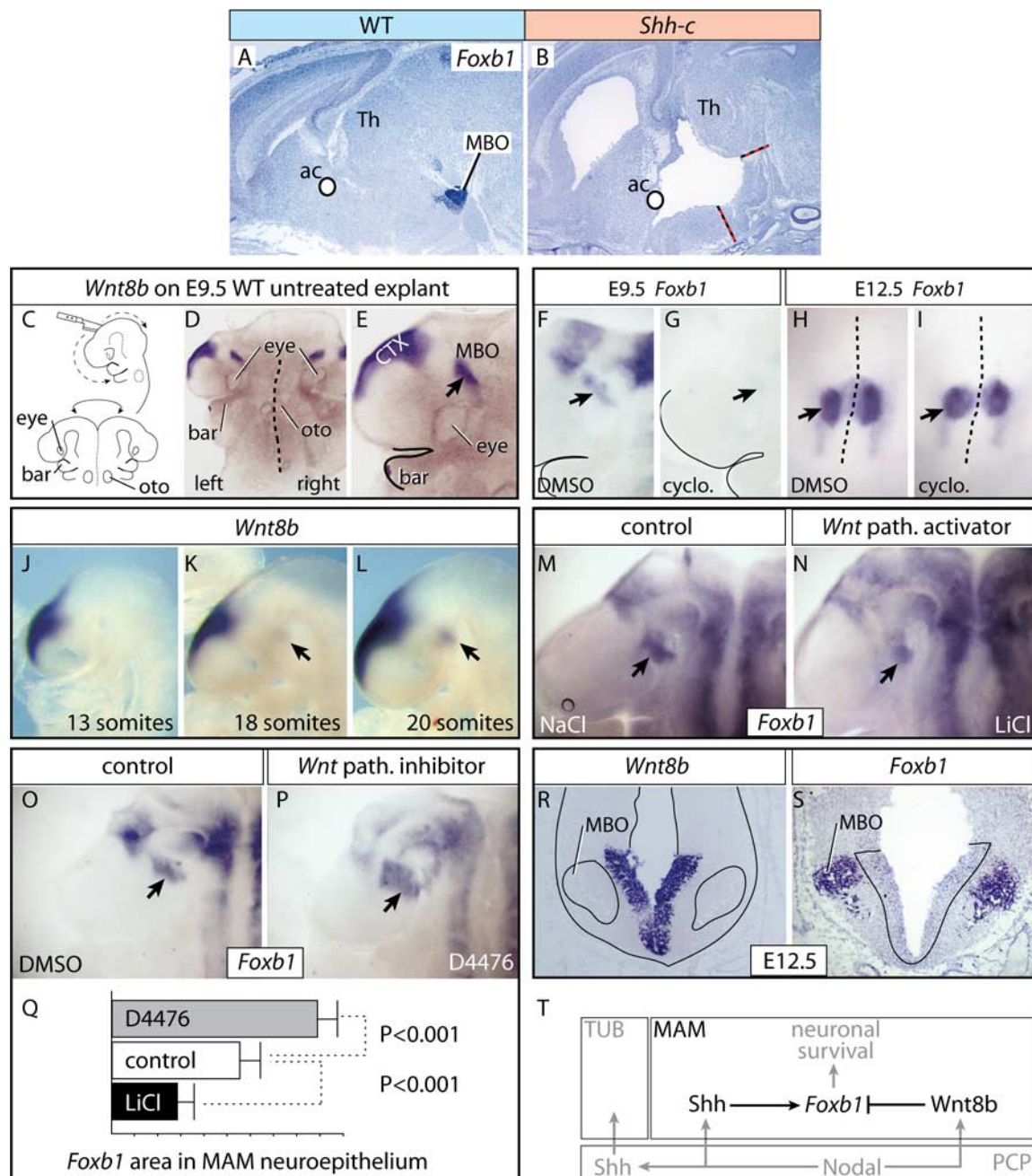


Figure 8. Neural *Shh* and Wnt pathway maintain and restrict expression of MAM survival factor *Foxb1*. **A, B**, *Foxb1* expression on sagittal sections of wild-type (**A**) and *Shh-c* (**B**) E18.5 brains. The specific expression in the MBO (**A**) is not present in the mutant (**B**). **C**, Diagrams showing forebrain explant preparation. **D, E**, Appearance of the E9.5 mouse brain explants at lower (**D**) and higher (**E**) magnification. This explant is labeled for *Wnt8b*. **F–I**, Treatment with *Shh* pathway blocker cyclopamine at E9.5 eliminates *Foxb1* expression in the forebrain including MAM (arrows; compare **F, G**). At E12.5, there is no effect on the MBO (arrows; compare **H, I**). **J–L**, Development of *Wnt8b* expression in the MAM. Expression is absent at 13 somites (**J**), weakly detectable at 18 somites (**K**, arrow), and fully developed at 20 somites (**L**, arrow). **M, N**, Treatment with Wnt pathway (path.) activator LiCl reduces the *Foxb1* expression domain in the MAM neuroepithelium (arrow). **O, P**, Treatment with Wnt pathway inhibitor D4476 increases the domain of *Foxb1* expression in the MAM neuroepithelium (arrow). **Q**, The *Foxb1*-expressing MAM neuroepithelial surface area (in arbitrary units) was significantly larger after treatment with D4476 and significantly smaller after treatment with LiCl. Control explants cultured with DMSO. Shown are mean \pm SD. **R, S**, As *Wnt8b* expression (**R**) increases in the MAM neuroepithelium, *Foxb1* (**S**) decreases and becomes restricted to the mantle layer. The section boundaries have been outlined in **R**, and the *Wnt8b* domain in **S**. **T**, Diagram showing the control of *Foxb1* expression in the MAM. In black is shown our data, and in gray, data from the literature.

in progenitors (neuroepithelium), and therefore it counterbalances or modulates the positive effect of *Shh* in *Foxb1* expression maintenance. The Wnt ligand involved here could be *Wnt8b*, a specific marker of the MAM in all vertebrates (Cui et al., 1995; Hollyday et al., 1995; Kelly et al., 1995; Lako et al., 1998; Richardson et al., 1999; Garda et al., 2002; Houart et al., 2002). *Wnt8b* downregulates *Foxb1* in the MAM of zebrafish (Kim et al., 2002), controls mammillary neurogenesis (Lee et al., 2006), and could

be a local organizer (Erter et al., 2001; Houart et al., 2002). The interplay *Shh*/*Wnt* in *Foxb1* control is presumably downstream the Nodal pathway (Strähle et al., 1996; Erter et al., 2001; Rohr et al., 2001; Houart et al., 2002; Mathieu et al., 2002) (Fig. 8T).

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